Crucial Roles of MZF1 and Sp1 in the Transcriptional Regulation of the Peptidylarginine Deiminase Type I Gene (*PADI1*) in Human Keratinocytes

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Peptidylarginine deiminases (PADs) catalyze the conversion of protein-bound arginine residues into citrulline residues in a calcium-dependent manner. The PAD1 gene (*PADI1*) is expressed in a few tissues, including the epidermis, where the protein is detected with a higher level in the more differentiated keratinocytes. Using quantitative reverse transcription-PCR experiments, we show that *PADI1* mRNAs are more abundant in keratinocytes cultured with 1.2 than 0.15 mM calcium. We cloned and characterized the promoter region using human keratinocytes transfected with variously deleted fragments of the 5′-upstream region of *PADI1* coupled to the luciferase gene. We found that as few as 195 bp upstream from the transcription initiation site were sufficient to direct transcription of the reporter gene. Mutations of MZF1- or Sp1-binding sites markedly reduced *PADI1* promoter activity. Chromatin immunoprecipitation assays revealed that MZF1 and Sp1/Sp3 bind to this region *in vivo*. Furthermore, MZF1 or Sp1 small interfering RNAs (siRNAs) effectively diminished *PADI1* expression in keratinocytes cultured in both low- and high-calcium-containing medium. In addition, the expression of MZF1 and PAD1 increased in parallel when normal human epidermal keratinocytes underwent differentiation. These data indicate that MZF1 and Sp1/Sp3 binding to the promoter region drive the *PADI1* expression.

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INTRODUCTION

Peptidylarginine deiminase (PAD; EC 3.5.3.15) is a post-translational modification enzyme that catalyzes the conversion of protein-bound arginine residues into citrulline residues (protein deimination) in the presence of calcium ion. This modification significantly alters the charge of residues from positive to neutral, probably resulting in the unfolding of target proteins. Although the exact physiological functions of protein deimination have not been clarified, keratins, filaggrin, and trichohyalin have been shown to be deiminated during the terminal stages of epidermal differentiation (Resing *et al.*, 1995; Ishida-Yamamoto *et al.*, 1997). Moreover, an abnormally decreased level of deiminated keratin K1 has been reported in the involved areas of the epidermis of psoriatic patients (Ishida-Yamamoto *et al.*, 2000). Enhanced deimination of myelin basic protein has been observed in the brain of multiple sclerosis patients (Kim *et al.*, 2003) and in primary open-angle glaucoma (Bhattacharya *et al.*, 2006) and was also found in fibrin/vimentin in the synovial membrane of rheumatoid arthritis patients (Nijenhuis *et al.*, 2004; Vincent *et al.*, 2005). These facts suggested that protein deimination plays an important role in normal tissue physiological processes and is involved in some human diseases (Vossenaar *et al.*, 2003; Chavanas *et al.*, 2006).

Since the early reports of PAD activities in the hair follicles and epidermis of the mammals (Rogers and Taylor, 1977; Fujisaki and Sugawara, 1981), five isoforms of PAD have been identified in mammalian tissues and described on the basis of their cDNA nucleotide sequence and the *in vitro* biochemical features of their products (Vossenaar *et al.*, 2003; Chavanas *et al.*, 2006). Among them, three PAD isoforms (PAD1, PAD2, and PAD3) are reported to be expressed in human epidermis (Kanno *et al.*, 2000; Ishigami *et al.*, 2002; Guerrin *et al.*, 2003); two of them (PAD1 and PAD3) have also been detected in hair follicles (Terakawa

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Abbreviations: ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NHEK, normal human epidermal keratinocyte; PAD, peptidylarginine deiminase; RT, reverse transcription; siRNA, small interfering RNA

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et al., 1991; Nachat *et al.*, 2005a). We have reported the precise cellular localization of the three isoforms in human and murine epidermis and in human anagen hair follicles by confocal and immunoelectron microscopy analyses with highly specific antipeptide antibodies (Méchin *et al.*, 2005; Nachat *et al.*, 2005a, b). PAD1 was detected in the entire epidermis with an increasing intensity gradient from the basal to the granular layer. PAD2 was detected mainly in both the spinous and the granular layers with a more intense staining of the latter, whereas PAD3 expression was shown to be restricted to the granular layer and lower stratum corneum. PAD1 and PAD3 were also detected in the inner root sheath of the hair follicles, whereas PAD3 was the only isoform expressed in the medulla (Rogers *et al.*, 1997; Kanno *et al.*, 2000; Nachat *et al.*, 2005b).

These different patterns of location strongly suggest that the expression of each PAD gene (*PADI* gene) is tightly controlled during keratinocyte differentiation. To understand the regulatory mechanisms involved, we are currently focusing on the characterization of the proximal promoters of the human *PADI* genes and on transcription factors bound to these control elements in human keratinocytes. Our previous studies revealed that Sp1/Sp3 function as *cis*-acting factors of the human *PADI2* gene, whereas the Sp1/Sp3 and NF-Y transcription factors cooperate to control the transcription of *PADI3* (Dong *et al.*, 2005, 2006). In this study, we report on the identification of the minimal promoter of *PADI1* and *cis*-acting elements involved in the regulation of its expression. Our results indicate that MZF1 and Sp1 are critical for the expression of *PADI1* in keratinocytes.

RESULTS

PADI1 expression in cultured human NHEKs

To investigate the transcriptional regulation of *PADI1* in the epidermis, we initially analyzed its expression by real-time reverse transcription (RT)-PCR on total RNAs isolated from primary normal human epidermal keratinocytes (NHEKs) cultured under low (0.15 mM) or high (1.2 mM) concentration of Ca^{2+} ion and from HeLa cells. As shown in Figure 1, *PADI1* mRNA was detected in NHEKs, with a higher amount in keratinocytes cultured under high concentration of Ca^{2+}. Low expression levels of *PADI1* mRNA were detected in HeLa cells. These results strongly suggest that a high concentration of Ca^{2+} stimulates the expression of *PADI1* in NHEKs, and that NHEKs are suitable for the study of transcriptional regulation of the human *PADI1* gene.

Identification of the transcription initiation site

Primer extension with the protected RNA segments indicated that the transcriptional start site was 83 bp upstream of the translation initiation codon (ATG) (Figure S1). This is consistent with the fact that the sequence 5′-CACACTT-3′ at −84 to −78 bp upstream of the translational initiation site exactly matches the consensus sequence 5′-C(A/T)(G/T/C)(T/C/A)(C/T)(T/G/C)(T/C)-3′, where the first ''A'' indicates the transcriptional start site frequently used in human genes (Bucher, 1990). It also corresponds to the 5′-end sequence of *PADI1* cDNA (GenBank accession no. AB033768), which was determined by the 5′-RACE method (Guerrin *et al.*, 2003). Therefore, we determined that the transcriptional start site ''A'' of *PADI1* (referred to as +1) was at position 83 bp upstream of the translation initiation codon.

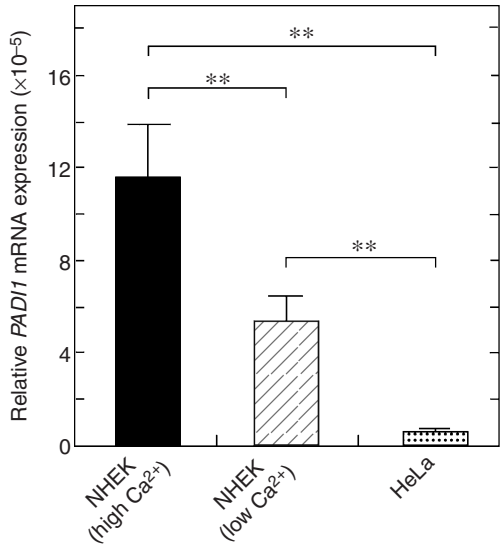

Figure 1. Expression of *PADI1* gene transcripts in NHEKs cultured in high (1.2 mM) or low (0.15 mM) Ca^{2+} ion-containing medium and in the HeLa cell line. Total RNAs were obtained from semiconfluent cell cultures. The relative *PADI1* mRNA levels were determined in cDNA samples by quantitative real-time PCR as described in the Materials and Methods section. Data were normalized to the glyceraldehyde-3-phosphate dehydrogenase gene. Results are means ± SD for four separate experiments. $^{**}P<0.01$ (Student's *t*-test).

Promoter activity of the 5′-flanking region of *PADI1* in cultured cells

To define the minimal promoter of *PADI1*, the 5′-flanking region and deletion mutants were cloned into the firefly luciferase reporter vector *pGBasic*. Each resulting recombinant plasmid was then transiently transfected into cultured keratinocytes and the luciferase activities were recorded after 48 hours (Figure 2a). To define putative differentiation-responsive regions within the promoters, NHEKs were incubated in either low- or high-calcium-containing medium. Higher luciferase activities were detected in NHEKs, as compared with HeLa cells, with the construct corresponding to the 5′-flanking region of *PADI1* (*pGB1*−2708/+84). Interestingly, a twofold increase in the activity was observed when NHEKs were shifted from low to high calcium concentration (Figure 2a). These results are consistent with the real-time PCR data reported above. They indicate that the *in vitro* −2708/+84 region is sufficient to drive the expression of *PADI1* and responds to conditions promoting differentiation of NHEKs. When the deletion mutants were tested, the luciferase activities of the *pGB1*−195/+84 construct were comparable to those of the constructs from *pGB1*−506/+84 to *pGB1*−2708/+84. Further deletion to −106 bp strongly reduced the luciferase activities under all tested conditions (Figure 2a). These results demonstrated that the −195/+84 region contained the core promoter for *PADI1* gene transcription and was calcium-responsive.

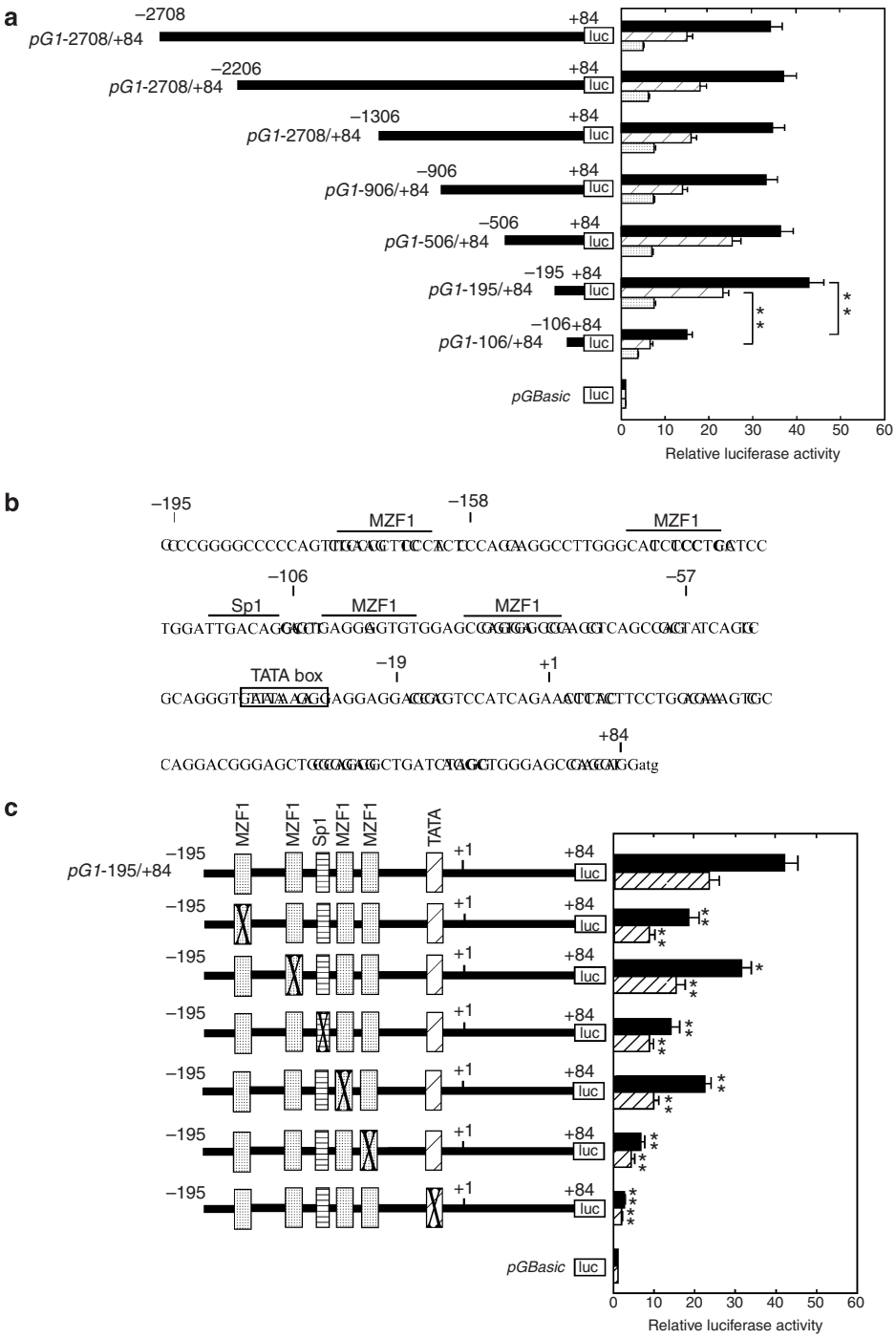

Figure 2. Identification and characterization of the minimal promoter of the *PADI1* gene. (**a**) Identification of the minimal promoter of *PADI1*. NHEKs cultured in high- (black bar) and low- (hatched bar) calcium-containing medium and HeLa cells (dotted bar) were transfected with the indicated constructs and assayed for luciferase activity after 48 hours. The numbers given to the constructs indicate the 5′ and 3′ ends of the 5′-flanking region of *PADI1*, the position numbered +1 corresponding to the transcription initiation site. Luciferase activity is expressed as fold increase over promoterless vector, *pGBasic* (set as 1). Values were normalized for transfection efficiency by cotransfection with the *Renilla* expression plasmid and are expressed as means±SD for four separate experiments. $^{**}P<0.01$ (Student's *t*-test). (**b**) Sequence and putative transcription factor-binding sites of the minimal promoter of *PADI1*. Position +1 refers to the transcription initiation site. Putative transcription factor-binding sites predicted by searching the BIOBASE P-Match-public v1.0 are underlined. (**c**) Characterization of the transcription factor-binding sites in the *PADI1* promoter by site-directed mutagenesis. Schematic diagram of serial deletion constructs of the *PADI1* promoter and their luciferase activities in NHEKs cultured in high- (black bar) and low- (hatched bar) calcium-containing medium is shown. The numbers given to the constructs indicate the 5′ and 3′ ends of the *PADI1* promoter region. The targeted putative transcription factor-binding sites are shown in the constructs with a solid cross. Site-directed mutagenesis was carried out with the construct spanning the −195 to +84 nucleotides. Values were corrected for transfection efficiency by cotransfection with the *Renilla* expression plasmid and are expressed as means±SD for four separate experiments. $^{*}P<0.05$, $^{**}P<0.01$ *versus* the difference between luciferase activity in *pG1*−195/+84 and mutant (Student's *t*-test).

Furthermore, *pGB1*−506/+84 exhibited the highest activity among the *PADI1* 5′-flanking region deletions in NHEKs under low-calcium concentration, and its activity increased by about 40% compared with *pGB1*−195/+84 (Figure 2a). This suggests that some enhancer elements are present between the −506 and −195 regions.

Searching the transcription factor database of BIOBASE P-Match-Public, numerous putative transcription factor-binding sites were identified in the 5′-flanking region of *PADI1*. In particular, within the −195/+84 region near the position of the transcriptional start site are sequences that closely match the consensus sequences recognized by MZF1 (four binding motifs) and Sp1 (one binding motif), suggesting that these transcription factors are involved in the basic regulation of *PADI1* promoter activity (Figure 2b). Furthermore, luciferase activities were significantly reduced by mutation of an MZF1- or the Sp1-binding site compared with the original plasmid. Mutation of the TATA box completely abolished *PADI1* promoter activity (Figure 2c). These results strongly suggest that the MZF1- and Sp1-binding sites and the TATA box found between nucleotides −195 and +84 are cooperative *cis*-elements critical for *PADI1* promoter activity.

Binding of transcription factors to the *PADI1* minimal promoter

To test whether the predicted transcription factors actually bind to the *PADI1* promoter *in vivo,* we performed a chromatin immunoprecipitation (ChIP) assay using specific antibodies, specific primers for the *PADI1* promoter region, and formaldehyde-fixed chromatin isolated from cultured cells. As expected, Sp1 and MZF1 bound to the 5′-flanking region of *PADI1*. Sp3 is coexpressed with Sp1 in several tissues/cell types and recognizes the same DNA element (Suske, 1999). Therefore, we also performed the ChIP assay with Sp3-specific antibody and showed the binding of Sp3 to the proximal promoter region of *PADI1* (Figure 3a, panel 1). When PCR was performed on the chromatin samples immunoprecipitated with nonimmune IgG using the same primers, no PCR signal was observed, showing the specificity of binding. The input levels of chromatin for each cell line were shown using nonimmunoprecipitated DNA as templates for PCR. Further specificity of the ChIP analysis was shown by the inability to detect binding of Sp1/Sp3 or MZF1 to exon 2 of *PADI1* (Figure 3a, panel 2). To quantify Ca^{2+} ion effects on the transcription factor binding, we carried out quantitative PCR with the immunoprecipitated chromatin obtained from NHEKs cultured in low- or high-calcium-containing medium. As shown in Figure 3b, higher levels of Sp1 and MZF1 recruitment were observed with high Ca^{2+} treatments. Interestingly, Sp3 binding was lower when the cells were cultured with high than with low Ca^{2+} concentration. These findings suggest that the association of MZF1 and Sp1/Sp3 on the promoter region of *PADI1* may play a prominent role in the transcription of the gene.

Expression of Sp1 and MZF1 is essential for *PADI1* expression in NHEKs

We carried out siRNA experiments to investigate the roles of Sp1, Sp3, and MZF1 *in vivo* in *PADI1* gene regulation. As

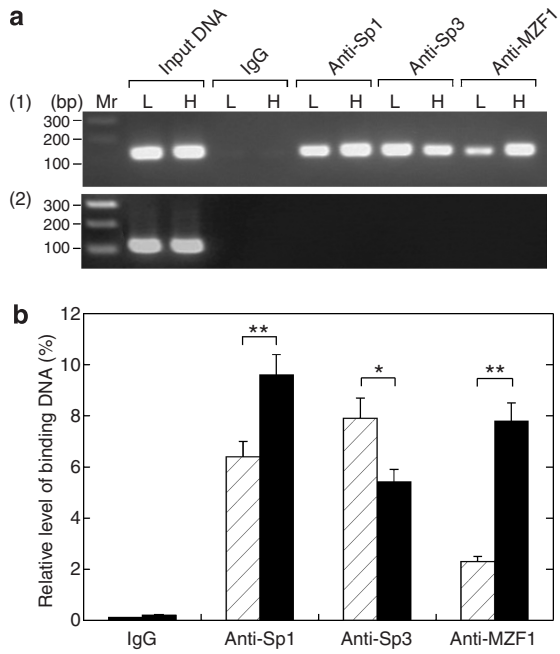

Figure 3. Sp1, Sp3, and MZF1 binding to the *PADI1* promoter *in vivo*. ChIP assays using anti-Sp1, anti-Sp3, or anti-MZF1 antibodies and without any antibodies were performed using chromatin from NHEKs cultured in low- (L) or high- (H) calcium-containing medium as described in the Materials and Methods section. (**a**) Sp1, Sp3, or MZF1 binding to the *PADI1* promoter was detected by gel staining after PCR amplification using primers corresponding to the promoter region (panel 1) and exon 2 (panel 2, negative control) of *PADI1*. Sizes are given in bp. (**b**) For quantitative analyses of Sp1, Sp3, and MZF1 binding to the *PADI1* promoter, the samples from NHEKs cultured in low- (hatched bar) and high- (black bar) calcium-containing medium were analyzed using real-time PCR. The relative DNA levels were calculated as described in the Materials and Methods section. Results are means±SD for four separate experiments. *$P<0.05$, **$P<0.01$ (Student's *t*-test).

shown in Figure 4a, the siRNAs transferred hindered the synthesis of each transcription factor by NHEKs in a specific manner. When Sp1 expression was reduced by specific siRNA transfection, the level of *PADI1* transcription in the cells cultured in both low- and high-calcium-containing medium was diminished significantly by more than 40% (Figure 4b). When MZF1 expression was reduced by a specific siRNA, the level of *PADI1* expression in NHEKs cultured under both conditions was decreased by more than 30% (Figure 4b). However, when the Sp3 expression level was effectively silenced by Sp3-specific siRNA, the levels of *PADI1* expression were not significantly affected (Figure 4b). Control siRNA did not significantly affect *PADI1* expression. These data demonstrate that Sp1 and MZF1 are necessary for the transcription of *PADI1* in NHEKs.

High calcium induces NHEK differentiation and expression of PAD1 and MZF1

To investigate the effects of high calcium on the keratinocyte differentiation and expression of PAD1 and MZF1, we carried out Western blot analyses of the total proteins extracted from 1.2 mM Ca^{2+}-treated NHEKs and 0.15 mM Ca^{2+}-treated

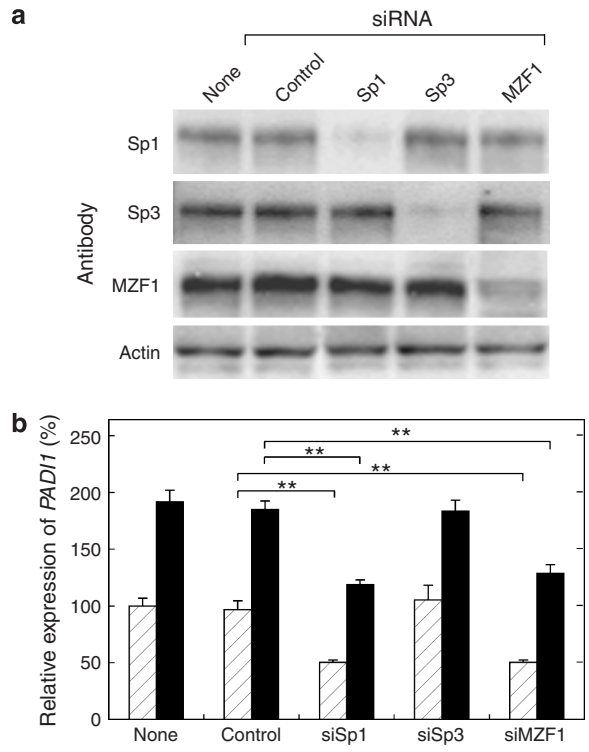

Figure 4. Binding of Sp1 and MZF1 to the *PADI1* promoter is essential for *PADI1* expression in NHEK. NHEKs were transfected with the indicated siRNA at 100 nM and cultured for 36 hours. Nuclear proteins and total RNA were extracted for Western blotting with the indicated antibodies and quantitative real-time PCR analysis, respectively. (**a**) Inhibition of expression of Sp1, Sp3, and MZF1 with their respective specific siRNAs was confirmed by Western blotting, as compared with control siRNA treatments and with treatments without any siRNA (none). The levels of the nuclear isoform of actin were used as loading controls. (**b**) Effects of inhibition of Sp1, Sp3, and MZF1 on *PADI1* expression. *PADI1* expression level in NHEKs cultured in low- (hatched bar) and high- (black bar) calcium-containing medium was normalized to the level of expression of glyceraldehyde-3-phosphate dehydrogenase and expressed relative to the nontreated NHEKs cultured in low-calcium-containing medium. Results are means ± SD for four experiments. $^{**}P < 0.01$ (Student's *t*-test).

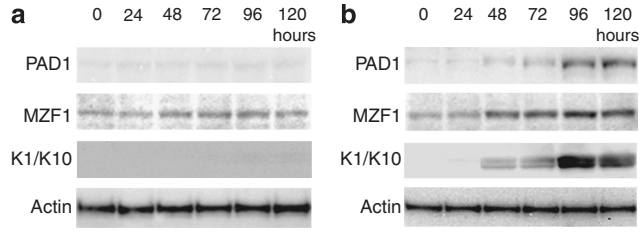

Figure 5. High calcium induces NHEK differentiation and expression of PAD1 and MZF1. NHEKs were cultured in 0.15 mM calcium-containing medium as control (**a**) or exposed to 1.2 mM calcium (**b**) for the indicated length of time. Proteins were extracted with SDS buffer and analyzed by Western blotting using anti-PAD1, anti-MZF1, or anti-K1/K10 antibodies. K1 and K10 were used as differentiation marker. Actin was used as a loading control.

NHEKs as control. As shown in Figure 5, the expression of K1 and K10, two markers of keratinocyte differentiation, was detected from 48 hours of high-calcium induction and increased to a maximum level at 96 hours. In a similar way, the level of PAD1 gradually increased after stimulation with high calcium. The expression level of MZF1 was significantly increased after 48 hours of high-calcium induction, up to a maximum level after 96 hours, supporting its role in upregulating the *PADI1* transcription. Neither K1/K10 expression nor changes in PAD1 or MZF1 detection level were clearly observed using control cells cultured in low-calcium-containing medium.

DISCUSSION

In this study, the regulatory mechanisms of *PADI1* gene expression were studied by cloning and functional characterization of the promoter region in human keratinocytes. One putative Sp1- and four putative MZF1-binding sites showed significant effects on *PADI1* promoter activity. Further comparison of regions of about 200 nt upstream of the transcription initiation sites of *PADI1* and *PADi1*, the mouse orthologous gene, revealed extensive identities including these putative binding sites (Figure S2). On the basis of these facts, we suspected these conservative *cis*-elements could function as key regulators for the basal expression of the *PADI1* gene in mammalian tissues. Moreover, similar to the increased detection of PAD1 in the epidermis throughout the course of differentiation (Nachat *et al.*, 2005b), we showed that the expression of *PADI1* was enhanced at both the transcriptional and the protein level in NHEKs cultured in 1.2 mM calcium as compared to 0.15 mM. This suggests that NHEKs might be an excellent system *in vitro* for studying the molecular basis of *PADI1* gene regulation (Figures 1 and 5).

We demonstrated that Sp1 bound to the *PADI1* promoter *in vivo* (Figures 3b and 4). Sp1 is the prototypical member of the Sp/Krüppel-like family of zinc-finger proteins that function as transcription factors in mammalian cells (Kaczynski *et al.*, 2003). Since its identification in 1983 (Dynan and Tjian, 1983), Sp1 has been shown to regulate many genes and may take part in virtually all facets of cellular function, including cell proliferation, apoptosis, and differentiation (Suske, 1999). It was originally identified as a ubiquitous transcription activator that was implicated in the constitutive expression of a considerable number of genes, including *PADI2* and *PADI3* (Dong *et al.*, 2005, 2006). Sp1 regulates the transcription of a number of TATA-containing promoters, often coordinating with other cellular transcription factors depending on which promoter they bind to and the coregulators with which they interact in a tissue-, cell-, and promoter-specific manner (Suske, 1999; Kaczynski *et al.*, 2003; Lomberk and Urrutia, 2005; Douet *et al.*, 2007). Moreover, recent studies have revealed the existence of an Sp family, with Sp1 and Sp3 being characterized extensively and known to be coexpressed in several tissues/cell types and to interact with an identical consensus sequence (Suske, 1999; Safe and Abdelrahim, 2005). Furthermore, Sp3 expression can either activate or repress promoter activity, depending on the promoter and type of cell (Pagliuca *et al.*, 2000; Ammanamanchi and Brattain, 2001; Ammanamanchi *et al.*, 2003). In human epidermis, Sp1 is an important regulator of genes involved in epidermal differentiation, including those

of keratin 5, involucrin, loricrin, transglutaminase (Nakamura *et al.*, 2007), and PAD2 and -3 (Dong *et al.*, 2005, 2006). In our study, ChIP assays indicated that the amounts of Sp1 and Sp3 recruitment to the *PADI1* promoter and the Sp1/Sp3 ratio changed according to the concentration of calcium in the NHEK culture medium, in a way consistent with the levels of *PADI1* mRNA detected by real-time RT-PCR. This suggests that the ratio of Sp1 and Sp3 binding may be responsible for the induced expression of PAD1 in the upper keratinocyte layers of epidermis. The calcium gradient observed in the tissue is known to be involved in keratinocyte differentiation (Hennings *et al.*, 1980). However, reduction of Sp3 expression using specific siRNA did not significantly influence the level of basal and Ca^{2+}-enhanced *PADI1* expression (Figure 4b). Therefore, it is possible that the absence of Sp3 could be compensated for by Sp1, but not the reverse, or that the remaining amount of Sp3 after siRNA treatment is still sufficient to regulate the transcription of *PADI1*, or that other transcription factors may be involved.

Our results (Figures 3–5) also strongly indicated the binding of MZF1 to the core promoter region of *PADI1* and the involvement of this transcriptional factor of the Krüppel family in accounting for the differentiation-specific expression of *PADI1*. Interestingly, MZF1-binding sites have not been identified in the promoter regions of *PADI2* and *-3* (Dong *et al.*, 2005, 2006). MZF1 was found to play a key role in cell lines representing early stages of myeloid differentiation and derivation of ES cell lines (Perrotti *et al.*, 1995) involved in growth, differentiation, and apoptosis of myeloid progenitors and regulating transcription during differentiation along the myeloid lineage (Bavisotto *et al.*, 1991; Hromas *et al.*, 1991; Perrotti *et al.*, 1995). Moreover, MZF1 is reported to be a bifunctional transcription regulator, containing 13 C_2H_2 zinc-finger domains divided into two groups that can bind DNA independently, depending on the cellular environment (Hromas *et al.*, 1996; Le Mee *et al.*, 2005). However, so far, the function of MZF-1 in transcription regulation has been unexplored in human keratinocytes. Thus, *cis*-acting motifs of MZF1 in transcriptional regulation in keratinocytes will be of future interest. We found that two of the four MZF1-binding sites (nt positions −73 and −163) were clearly the most important in the response to high Ca^{2+} treatment, especially the most proximal to the TATA box. This site was also the most confident, with a *P*-value of 1.0 in the *in silico* motif search. These observations suggested that the two sites might be critical as two main binding sites of MZF1. In addition, the other two MZF1 sites on either side of the Sp1-binding site also retained certain calcium sensitivity and may ensure adequate expression of *PADI1*. It could be that MZF1 binding to these two sites may be weaker due to the binding of and blocking by Sp1. However, we also cannot exclude the possibility that a cooperation between MZF1 and Sp1 may exist in specific cell or tissue types or in response to a specific condition. MZF1 probably acts as an activator of the basic transcriptional activity during cell proliferation, which in response to extracellular Ca^{2+} initiate signaling cascades that lead to the promotion of *PADI1* expression during early cell differentiation (Figure 5).

As the extracellular Ca^{2+} concentration in the epidermis increases from the basal layer to the stratum granulosum (Bikle *et al.*, 2004), it is likely that the level of Ca^{2+} results in *PADI1* gene regulation in the different keratinocyte layers through the distribution/activity of transcription regulators such as Sp1/Sp3 and MZF1, which may direct keratinocyte-specific and differentiation-specific expression.

The five PAD genes share significant similarity in their organization and have evolved from a common ancestral gene (Chavanas *et al.*, 2004). However, the nucleotide sequences of their 5′-flanking regions are not conserved, and mechanisms for their regulation seem to be diverse. Indeed, *PADI2* is widely expressed in a variety of tissues (Ishigami *et al.*, 2002), whereas other *PADI* genes are tissue-specifically expressed (Chavanas *et al.*, 2006). *PADI2* contains a TATA-less but GC-rich 5′-flanking region (Dong *et al.*, 2005), whereas *PADI1* (this study), *PADI3* (Dong *et al.*, 2006), and *PADI4* (Dong *et al.*, 2007) are likely to be regulated by TATA-box-containing promoters. Interestingly, in our previous studies, we discovered that Sp1/Sp3 was involved in the regulation of *PADI2*, *-3*, and *-4* gene expression (Dong *et al.*, 2005, 2006, 2007), whereas NF-Y was involved in one of either *PADI3* or *-4* (Dong *et al.*, 2006, 2007). It might be one of the key issues that the level of Ca^{2+} changing within the different keratinocyte layers in human epidermis results in *PADI* gene regulation through the distribution/activity of transcription regulators such as Sp1, Sp3, MZF1, and NF-Y.

In conclusion, the identification of the human *PADI1* minimal promoter and of transcription factors bound onto it, as presented in this study, will help to identify the regulatory mechanisms involved in tissue- and differentiation stage-specific expression of the human PAD1 genes and, more generally, in the control of the complex differentiation program of keratinocytes necessary for human skin homeostasis or pathology. As the importance of PADs in skin and other tissue diseases such as multiple sclerosis and rheumatoid arthritis is becoming clearer (Kim *et al.*, 2003; Sebbag *et al.*, 2004; Chavanas *et al.*, 2006), it is crucial to investigate the mechanisms of their accurate regulation, including transcriptional and translational controls, which may pave the way to regulating abnormal deimination, taking PADs as possible therapeutic targets in the future.

MATERIALS AND METHODS

All experiments were conducted in accordance with the Declaration of Helsinki Principles and were approved by the Ibaraki University Ethical Committee of Human Genome and Gene Research.

Cell cultures

NHEKs were obtained from Clonetics (San Diego, CA) and cultured in KGM2 (Clonetics, San Diego, CA) with either 0.15 mM (proliferating conditions) or 1.2 mM Ca^{2+} (differentiating conditions) as described previously (Hennings *et al.*, 1980). Unless specifically indicated, NHEKs were exposed to 1.2 mM Ca^{2+} for 96 hours to induce the differentiation. HeLa cells were obtained from Health Sciences Research Resources Bank (Osaka, Japan) and maintained in DMEM (Gibco-BRL, Rockville, MD) with 10% (v/v) fetal bovine

serum (HyClone Laboratories, Logan, UT). All the cells were incubated at 37°C with 5% CO_2. After reaching 70–80% confluence, the cells were collected for subculture or transfection.

Real-time RT-PCR analysis

The relative expression values of *PADI1* were analyzed by real-time RT-PCR, as described previously (Dong *et al.*, 2005). The following primer pair was used to amplify a 104 bp fragment of *PADI1* exon 2: a forward primer, 5′-GTCTACAACCGCACACGT-3′ (positions 240–257 of the human PAD1 cDNA: GenBank accession no. AB033768), and a reverse primer, 5′-AATTCCTTACTGGCTGTGC-3′ (positions 343–325). The amount of cDNA was normalized in PCR controls with primers specific to glyceraldehyde-3-phosphate dehydrogenase: forward primer, 5′-CATGTTCCAATATGATTCCAC-3′ (positions 187–207 of the human glyceraldehyde-3-phosphate dehydrogenase cDNA: accession no. M33197), and reverse primer, 5′-CCTGGAAGATGGTGATG-3′ (positions 271–287). Real-time quantitation was performed using the SuperScript™ III Platinum® Two-Step qRT-PCR with SYBR® Green Kit (Invitrogen, Carlsbad, CA) and iCycler IQ™ detection system (Bio-Rad, Hercules, CA). After denaturation at 95°C for 3 minutes, amplification was carried out for 50 cycles using a two-step protocol: at 95°C for 30 seconds and at 58°C for 30 seconds.

Cloning of the 5′-flanking region of *PADI1*

Based on the nucleotide sequences from the human genomic contig (GenBank accession no. NT 030584.7), the 5′-flanking region of *PADI1* was amplified by two-step PCR. In brief, the first PCR was performed with human genomic DNA (BD Biosciences, Palo Alto, CA) as a template and the specific human *PADI1* primer pair 5′-AGTCTTGCTCTGTTGC-3′ (forward: −2708 to −2693) and 5′-TGTGAATGTCCACATG-3′ (reverse: +158 to +174). The PCR conditions were 95°C for 2 minutes, 30 cycles (95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 4 minutes), and 72°C for 8 minutes with Ex Taq DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions. Subsequently, nested PCR was carried out with the resulting DNA fragments and forward primer G1-2708/2689 and reverse primer G1-RV (Table S1); the conditions were 95°C for 1 minute, 30 cycles (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 4 minutes), and 72°C for 8 minutes. The final products of the nested PCR were subcloned into the pGEM®-T vector (Promega, Madison, WI). The obtained plasmid, *pTAhPAD1,* covered a 2791-bp nucleotide sequence of the 5′ region of *PADI1*. Sequencing of each clone was carried out on an automated DNA sequencer (373 DNA Sequencer, Applied Biosystems, Foster City, CA).

RNase protection analysis

To determine the 5′ end of *PADI1* mRNA, RNase protection assays were performed as described before (Dong *et al.*, 2005). These procedures are provided in detail in the Supplementary Materials and Methods.

Searching the conserved noncoding segments and transcriptional motifs

The sequence of the 5′-flanking regions of mouse *Padi1* was obtained through searching the chromosome 4 contig AL645625. The program DNA Block Aligner (http://www.ebi.ac.uk/Wise2/dbaform.html) was used to analyze both the sequences of human and mouse (Jareborg *et al.*, 1999). The putative transcription factor-binding sites were characterized by searching the BIOBASE P-Match-Public v1.0 (http://www.gene-regulation.com/index.html; group of matrices was vertebrate; cutoffs for core and matrix similarity were 1.0 and 0.90, respectively).

Construction of promoter reporter plasmids

A *Kpn*I–*Bgl*II fragment (covering −2708 to +84 bp from the transcriptional start site) of *pTAhPAD1* was inserted into the *Kpn*I–*Bgl*II sites of *pGBasic* vector 2 containing the firefly luciferase gene (Nippon Gene, Toyama, Japan). Sequential 5′-deletion constructs of the 5′-flanking region of *PADI1* were generated by PCR using *pG1*-2708/+84 as a template and primers listed in Table S1. The thermocycler settings were 95°C for 2 minutes, 30 cycles (95°C for 30 seconds, 30 seconds at the predicted melting temperature of each forward primer, 72°C for 3 minutes), and 72°C for 10 minutes. The amplification products were ligated into the *Kpn*I–*Bgl*II sites of *pGBasic*. To construct promoter reporter plasmids with site mutations, PCR was performed using *pG1*-195/+84 as the template and the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The six pairs of oligonucleotides used in thermal cycling are listed in Table S1. All mutation constructs were confirmed by DNA sequencing.

Transfection and measurement of promoter activity

Transfection and measurement of promoter activities were carried out following procedures described previously (Dong *et al.*, 2005). Four transfections were carried out independently for each construct, and the results were expressed as means ± SD.

ChIP assays

ChIP assays were performed as described in the protocol of the Chromatin Immunoprecipitation Assay Kit (Upstate, Lake Placid, NY). Immunoprecipitation was performed overnight with agitation at 4°C with 5 μg of normal IgG, anti-MZF1, anti-Sp1, or anti-Sp3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitated chromatin was collected and analyzed by conventional PCR and quantitative PCR. The following primers were used for ChIP PCR analysis to amplify the promoter region from nucleotide −174 to −45: 5′-TCCCCTTCCCCATCTCCCA-3′ (forward; nucleotides −174 to −156) and 5′-CTGCGACCTGATTAGCCTGG-3′ (reverse; nucleotides −64 to −45). The amplification program consisted of denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. The control PCR for ChIP analysis was performed using the primers and conditions described above under ''Real-time RT-PCR analysis.''

siRNA-based inhibition

NHEKs were transfected using the siRNA transfection reagent (Santa Cruz Biotechnology) with 100 nM siSp1, siSp3, siMZF1, or siControl (Santa Cruz Biotechnology) according to the manufacturer's instructions. After the cells were cultured in antibiotic-free medium for 48 hours, total RNA was extracted and analyzed by real-time RT-PCR as described above. To confirm the specific inhibitory activity of each siRNA, we carried out Western blotting analyses with the antibodies against each transcription factor as described below.

Western blotting

Total proteins were extracted with SDS buffer (0.125 M Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, and 10% sucrose) and

sonicated on ice for 30 seconds. The nuclear extracts were prepared from untreated or siRNA-transfected cells as described previously (Dong *et al.*, 2005). Primary anti-PAD1 (Nachat *et al.*, 2005b), anti-K1/K10 (EE21-06, Barbaud *et al.*, 1998), anti-MZF1, anti-Sp1, anti-Sp3, or anti-actin antibodies (Santa Cruz Biotechnology) were added in skimmed milk solution at a sufficient dilution (1:200–1:10,000). The secondary anti-rabbit, anti-mouse, or anti-goat (Santa Cruz Biotechnology) antibodies were used at a dilution of 1:3,000, 1:5,000, or 1:12,000, respectively. The proteins were detected using the ECL (enhanced chemiluminescence) Plus Western blotting detection system (Amersham Pharmacia Biotech; Arlington Heights, IL).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Text. Supplementary Materials and Methods.

Table S1. Primers used for preparing the deletion mutants and oligonucleotides for motif mutants.

Figure S1. Determination of the transcription start site of the *PADI1* gene.

Figure S2. Sequence alignment of the promoter regions of human *PADI1* and mouse *Padi1* genes.

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